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ACTINOBACILLUS SEMINIS INFECTION IN SHEEP IN THE REPUBLIC OF SOUTH AFRICA. III. GROWTH AND CULTURAL CHARACTERISTICS OF *A. SEMINIS*

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ABSTRACT

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Bacteriological tests were done on a large number of different strains of *Actinobacillus seminis* and also, repeatedly, on the same culture or on different cultures taken periodically from the same donor animal. These tests were also applied to strains of *A. seminis* representing different serological types, which in turn were compared with strains of *Brucella ovis*.

The tests as applied proved that *A. seminis* strains have defined, morphological, staining, cultural and biochemical properties, although they can generally be regarded as biochemically inactive. Growth was greatly enhanced on media enriched with blood or serum and also more luxuriant when incubated in a carboxophilic atmosphere. Nitrate reduction was found to be a variable characteristic, as it was more often negative, while weakly positive and negative reactions for hydrogen sulphide production were encountered with equal frequency.

On the basis of their bacteriological properties, the strains representing the different serological types can be divided into 2 groups. Strains belonging to the first of these groups conform to the earlier description of *A. seminis* by Baynes & Simmons (1960) and are usually catalase positive and oxidase negative, while those in the second group more closely resemble *Histophilus ovis* described by Roberts (1956), and produce variable reactions on the catalase and oxidase tests.

Although growth did occur aerobically and was more luxuriant in a carboxophilic atmosphere in all strains, it was always much slower for strains resembling *H. ovis*. Similarly, the growth produced by these strains was poorer and more irregular on ordinary nutrient media and, although greatly enhanced and more regular in all strains on enriched media, it was again much slower for these strains. In all stages of development, the colonies of strains similar to *H. ovis* were always slower and more transparent in appearance, and tended to remain low convex and undifferentiated. Packed organisms of these strains were light yellow (lemon) in colour in contrast to strains resembling *A. seminis*, which had a greyish-white appearance.

A. seminis and *B. ovis* can clearly be distinguished on their morphology, Stamp staining reaction on both semen and culture smears, colonial morphology, the delayed colony development of *B. ovis* and sensitivity to dyes and antibiotics.

Résumé

INFECTION À ACTINOBACILLUS SEMINIS *CHEZ LE MOUTON EN RÉPUBLIQUE SUD-AFRICAINE. III. CROISSANCE ET CARACTÉRISTIQUES D'*A. SEMINIS *EN CULTURE*

*On a effectué des tests bactériologiques sur un grand nombre de souches différentes d'*Actinobacillus seminis *et aussi, à plusieurs reprises, sur la même culture ou sur des cultures différentes prélevées périodiquement sur le même animal. Ces tests ont également été appliqués à des souches d'*A. seminis *représentant différents types sérologiques et ces derniers ont à leur tour été comparés à des souches de* Brucella ovis.

*Les tests tels qu'on les a appliqués ont montré que les souches d'*A. seminis *ont des propriétés morphologiques, de coloration, en culture et biochimiques bien définies, quoiqu'on puisse de façon générale les considérer comme biochimiquement inactives. La croissance a été fortement augmentée sur des milieux enrichis de sang ou de sérum et a également été plus luxuriante quand les cultures étaient incubées dans une atmosphère carboxophilique. La réduction des nitrates s'est avérée être une caractéristique variable, étant le plus souvent négative. Par contre, en ce qui concerne la production d'hydrogène sulfuré, on a rencontré aussi souvent des réactions faiblement positives que des négatives.*

*Sur la base de leurs propriétés bactériologiques il est possible de partager en 2 groupes les souches qui représentent les divers types sérologiques. Les souches appartenant au premier de ces groupes vérifient l'ancienne description d'*A. seminis *par Baynes et Simmons (1960) et sont généralement catalase-positives et oxidase-négatives. Celles du second groupe ressemblent davantage à la description d'*Histophilus ovis *faite par Roberts (1956) et donnent des réactions variables aux tests de la catalase et de l'oxidase.*

Quoique la croissance fût aérobie pour toutes les souches et plus luxuriante dans une atmosphère carboxophilique, elle a toujours été beaucoup plus lente pour les souches resemblant à H. ovis. *Semblablement, la croissance de ces souches a été plus pauvre et plus irrégulière sur des milieux nutritifs ordinaires; et bien qu'elle ait été fortement augmentée et plus régulière sur milieux enrichis, elle a de nouveau été beaucoup plus lente pour ces souches-là. A tous les stades de développement, les colonies de souches ressemblant à* H. ovis *ont toujours été plus lentes et d'apparence plus transparente, tendant à rester bassement convexes et indifférenciées. La couleur des organismes entassés de ces souches était jaune clair (citron), à la différence des souches ressemblant à* A. seminis, *d'apparence gris-blanc.*

On peut nettement distinguer A. seminis *et* B. ovis *d'après leur morphologie, leur réaction à la coloration de Stamp sur frottis séminaux et sur frottis de cultures, leur morphologie coloniale, le retard au développement des colonies de* B. ovis *et la sensibilité aux colorants et antibiotiques.*

INTRODUCTION

Baynes & Simmons (1960) described certain morphological, cultural and biochemical properties of the first strain of *Actinobacillus seminis* to be isolated in Australia. In subsequent reports these characteristics, with minor differences, were confirmed by Livingston & Hardy (1964) and Worthington & Bosman (1968). All these findings, however, were based on single strains, with the result that possible variations between different isolates were not apparent.

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Worthington & Bosman (1968) found that their strain of *A. seminis* produced a clot but no acid in litmus milk, while the Australian strain described by Baynes & Simmons (1960) did not produce this change. In earlier investigations and those conducted in South Africa, no changes occurred in litmus milk with any of the cultures studied (Van Tonder & Bolton, 1968).

Although no comment was made regarding the reduction of nitrates by the overseas workers, Baynes & Simmons (1960) and Livingston & Hardy (1964), Worthington & Bosman (1968) showed that both

their strain and the Australian one reduced nitrates to nitrites. On the other hand, tests conducted in this laboratory on the 2 strains obtained from the Veterinary Research Institute, Onderstepoort, as well as on a large number of local strains, showed that the reduction of nitrates was a variable characteristic and was frequently negative. The shortcoming of this test for routine purposes was the fact that it was designed to determine the presence of nitrites only, and there was the possibility that this product might have been broken down further and be therefore no longer present in demonstrable quantities in those tests which had proved negative.

Certain minor differences in cultures from the same organism were observed from time to time, and it was considered expedient to undertake further bacteriological investigations to establish a more clearly defined pattern of growth requirements and biochemical properties.

This paper records the results of tests on a large number of different strains cultured on a comprehensive range of culture media. These tests were also applied to strains of *A. seminis* representing different serological types. To confirm the reproducibility of these tests or detect possible changes in cultural characteristics brought about by handling in the laboratory, repeated experiments were conducted periodically on the same culture or on different cultures taken from the same donor animal. The cultural behaviour of different serological strains of *A. seminis* was studied and compared with that of *B. ovis*, a well-defined and characterized pathogen of the male genital system.

MATERIALS AND METHODS

Bacteriological samples

Cloned cultures of *A. seminis* were prepared and selected from isolates derived from a variety of clinically affected rams and from others which showed neutrophils in their semen. In addition, 6 serologically different strains, designated T981V, 70.64, 6201, U207, 1032A and V350 (Van Tonder, 1977; Van Tonder, to be published), were included in the investigation. The majority of the tests were conducted on recently isolated strains. Where repeated tests were undertaken with the same sample, freeze-dried cultures were used.

A reference strain of *B. ovis* (6010) was obtained from Dr R. W. Worthington of the Veterinary Research Institute, Onderstepoort.

Bacterial culture and staining techniques

All the culture media and stains were prepared according to the instructions of the manufacturers of commercially available products.

Initially, primary cultures were made on nutrient agar (Alton & Jones, 1967) and Bacto blood agar base* containing 5% defibrinated horse blood. Subsequently, all primary cultures were made on Bacto tryptose blood agar* and/or *Brucella* agar** containing 5% defibrinated horse blood. In the earlier phase of these studies, the plates were incubated at 37 °C in an atmosphere of 10% carbon dioxide, but subsequently 20% of air was replaced by carbon dioxide. The latter method became the eventual standard procedure.

* Difco Laboratories, Detroit, Michigan, U.S.A.
** E. Merck, Darmstadt, Western Germany

The tests conducted to identify strains of *A. seminis* and to compare them with the reference strain of *B. ovis* included the following:

Morphology and the staining reactions were observed on smears prepared from cultures as well as from semen samples (Table 1).

Motility was assessed in wet preparations made from 24 and 48 h cultures and examined by phase-contrast microscopy at 22 °C and 37 °C, respectively.

Atmospheric requirements were determined by incubating the cultures under aerobic, anaerobic, micro-aerophilic as well as carboxophilic conditions. Anaerobiosis was created by evacuating and filling the anaerobic jars for at least 3 successive cycles with pure carbon dioxide (Table 2). Micro-aerophilic conditions were obtained by evacuating the jar containing the cultures until a vacuum of 55 cm of mercury was obtained. It was then filled with carbon dioxide and again evacuated to 55 cm of mercury before incubation (Simmons & Hall, 1953).

Growth characteristics were determined on a variety of ordinary, enriched and selective media (Table 3). A dilution of each sample was selected in order to yield an abundant growth of well-spaced colonies when seeded onto tryptose blood agar or *Brucella* agar plates enriched with 5% horse blood. Growth of the bacteria was observed and compared daily for a minimum period of 5 days, taking into account the abundance, size and rate of development of single colonies. Evaluation was based on the growth and size of individual colonies as well as on the rate of development as measured on the 5th day of incubation. This was recorded on a scale ranging from − to ++++, indicating no growth at all, to increasing abundance, with luxuriant and almost confluent growth.

The various biochemical and other tests conducted on strains of *A. seminis* are listed in Table 4. Nitrate reduction was determined by the method described by Cowan & Steel (1970) in which all negative tests were also tested for residual nitrates.

Inhibition of growth was evaluated by growing the various strains on serum dextrose agar containing antibiotics and/or selected dyes (Alton & Jones, 1967). The dyes were incorporated into the medium at the concentrations given in Table 5.

Sensitivity to various antibiotics was determined by placing Bacto unidiscs (high concentrations)* onto suitably prepared cultures. The antibiotics and concentrations used in these tests are given in Table 6. Growth was recorded as described above.

RESULTS

Morphological characteristics

Stained preparations showed the organisms to be non-sporulating, Gram-negative, pleomorphic and varying in shape from coccobacillary forms to rods. The longer forms varied from 4–5 μ in length, were approximately 1 μ in width and appeared singly or in pairs, in short or occasionally longer chains. They were non-acid-fast when stained by Stamp's modified technique. Organisms in semen smears occurred singly, in short chains and sometimes in palisade formation as small colonies. They were often found in close association with neutrophils or epithelial cells present in the semen. It was found that occasional

* Difco Laboratories, Detroit, Michigan, U.S.A.

intracellular organisms sometimes retained the carbol fuchsin and stained red with the modified Ziehl-Neelsen technique. All isolates of *A. seminis* proved to be non-motile. These results are summarized in Table 1.

Atmospheric requirements

Growth occurred aerobically on primary culture but was much slower for Strains 1032A and V350. When comparisons were made after 5 days incubation, growth was more luxuriant when cultures were incubated in a carboxophilic atmosphere in which 10% of air was replaced on carbon dioxide and, better still, in an atmosphere of 20% carbon dioxide. The latter became the standard isolation procedure (Table 2).

Growth characteristics

Growth was poor on primary culture, and irregular on ordinary nutrient media, and notably so on bacterial cultures, which were classified as strains serologically similar to 1032A and V350. On media enriched with blood or serum, primary growth was greatly enhanced and more regular, but again considerably slower with Strains 1032A and V350. In all subsequent studies, blood tryptose agar and *Brucella* agar enriched with horse blood were found to be the most suitable culture media for primary isolation and propagation of *A. seminis* (Table 3). Growth in all cases was poor in ordinary peptone water, but slightly improved when 5% inactivated horse serum was added. After 48 h incubation, the feeble growth could be observed as a stringy, occasionally granular, sediment, while the medium always remained clear.

On Bacto potato infusion agar, growth was variable and only some strains yielded a fine, almost transparent, growth.

No growth could be obtained on ordinary agar or MacConkey agar from any of these organisms.

Colony morphology

On 5% horse blood tryptose or *Brucella* agar, small, pin-point or slightly larger transparent colonies were observed after 24 h incubation, while the larger individual colonies had a typical "dewdrop-like" appearance. After 48 h incubation, colonies were up to 1–2 mm in diameter, were low convex or dome-shaped and round, with an entire margin and a greyish-white appearance. Strains 1032A and V350 showed slower development and their colonies were not only somewhat smaller at this stage but were also more transparent and dome-shaped.

After incubation for 3–4 days, colonies were up to 4 mm in diameter, umbonate, with greyish-white centres, having a transparent periphery and entire to undulate margins. The colonies of strains 1032A and V350 again were somewhat smaller, with a light and more transparent appearance, tending to remain low convex and undifferentiated. Another difference observed between these 2 and the other *A. seminis* strains was that packed organisms showed a light yellow (lemon) colour, whereas in the other strains they had a greyish-white appearance. This could best be observed by holding a loopful of growth against a white background. No haemolysis was observed in any of these cultures on the selected media, even after prolonged incubation.

TABLE 1 Morphology and staining characteristics of strains of *A. seminis* and *B. ovis*

Characteristics	*A. seminis* strains	*B. ovis*
Morphology:		
Shape	Pleomorphic coccobacilli up to 4–5 μ in length	Coccoid to short rods
Arrangement	Singly, in pairs, and in short chains	Singly, sometimes in pairs
Spore formation	Negative	Negative
Capsules	Negative	Negative
Staining reactions(1):		
Gram's method	Negative	Negative
Modified Ziehl-Neelsen technique	Negative	Positive
Ziehl-Neelsen technique	Negative	Negative
Hansen's method	Negative	Positive

(1) Semen and culture smears stained with:

Gram's method (Preston & Morrell, 1962)
The modified Ziehl-Neelsen technique (Stamp, *et al.* 1950)
Ziehl-Neelsen technique (Cruickshank, 1965)
Hansen's method (Van Drimmelen, 1960)

TABLE 2 Atmospheric requirements of strains of *A. seminis* and *B. ovis*

Item	Degree of bacterial growth[1]						
	A. seminis strains						*B. ovis*
	T981V	70.64	6210	U207	1032A	V350	
Aerobic	++	+++	+++	++	+	+	—
Anaerobic (air replaced by CO_2)	++	+	—	++	—	—	+
Carboxophilic (10% air replaced by CO_2)	++++	++	++++	+++	+++	++++	+++
Carboxophilic (20% air replaced by CO_2)	++++	++++	+++	++++	++++	++++	++++
Micro-aerophilic	++++	+++	++++	++++	++++	++++	++++

[1] — to ++++ = Degree of growth

TABLE 3 Bacterial growth obtained from strains of *A. seminis* and *B. ovis* on ordinary and enriched media

Item	Degree of bacterial growth						
	A. seminis strains						*B. ovis*
	T981V	70.64	6201	U207	1032A	V350	
Ordinary media:							
Agar plates[1]	—	—	—	—	—	—	—
Nutrient agar[1]	+	—	—	—	—	—	—
Dextrose nutrient agar[2]	++	++	++	++	—	—	—
Potato medium[3]	—	—	—	—	—	—	—
Alcaline potato[3]	—	—	—	—	—	—	—
Glycerol potato[3]	—	—	—	—	—	—	—
Potato infusion agar[2]	+	—	—	—	—	—	+++
Glycerol potato infusion agar[2]	—	+	+	—	—	—	++
Potato dextrose agar[4]	—	—	—	—	—	—	+
Glycerol dextrose agar[2]	+	+	+	+	—	—	—
Brucella agar[5]	+++	++	++	++	+++	+++	+++
Tryptocase soy agar[6]	+++	++	+++	++	++	++	++
Veal infusion agar[5]	++++	++	++	++	—	—	++
Liver infusion agar[5]	—	—	—	—	—	—	++
Brain heart infusion agar[5]	++++	++	+++	+	—	—	++
Tryptose agar[5]	+++	++	+++	+++	+	++	+++
Enriched media[7]:							
Nutrient agar & blood	+++	+++	++++	+++	—	++	+++
Nutrient agar & serum	+++	+++	+++	+++	—	+	+++
Dextrose nutrient agar & blood	++++	+++	++++	+++	+	+++	++++
Dextrose nutrient agar & serum	+++	+++	+++	+++	+	++	+++
Potato infusion agar & blood	+	+	+	+	—	—	++
Potato infusion agar & serum	—	+	—	—	—	—	+
Potato dextrose agar & blood	—	—	—	—	—	—	—
Potato dextrose agar & serum	—	—	—	—	—	—	—
Glycerol dextrose agar & blood	++	+++	++	++			
Glycerol dextrose agar & serum	++	++	++	++	—	+	++
Brucella agar & blood	++++	+++	+++	+++	+++	+++	++++
Brucella agar & serum	+++	++	+++	++	++	++	+++
Tryptocase soy agar & blood	++++	+++	++++	++++	+++	+++	++++
Tryptocase soy agar & serum	+++	+++	+++	+++	+++	++++	+++
Veal infusion agar & blood	+++	++	++	++	++	+++	++++
Veal infusion agar & serum	+++	++	++	++	++	++	+++
Liver infusion agar & blood	+++	+	+	++	+	++	++
Liver infusion agar & serum	+++	+	—	+	—	+	—
Brain heart infusion agar & blood	++++	+++	++++	+++	++	++++	+
Brain heart infusion agar & serum	+++	++++	++	++	—	+++	++
Tryptose blood agar & blood	+++	+++	+++	+++	+++	+++	+++
Tryptose blood agar & serum	+++	+++	+	+++	++	+++	+++

[1] Cowan & Steel (1970)
[2] Alton & Jones (1967)
[3] Silverton & Anderson (1961)
[4] Frankel, Reitman & Sonnenwirth (1970)
[5] Difco Laboratories, Detroit, Michigan, U.S.A.
[6] Baltimore Biological Laboratory Division, B-D Laboratories, Inc., Baltimore, Md., U.S.A.
[7] 5% horse blood or serum added

Biochemical reactions

None of strains examined produced acid or gas in the various media employed for detecting carbohydrate breakdown (Table 4) and all remained negative even after 14 days incubation. No change was observed in litmus milk, indole was not produced nor was urea decomposed, and gelatin was not liquefied.

None of the strains was able either to utilize sodium malonate and sodium citrate as sole sources of carbon, to oxidize potassium gluconate, to deaminate DL-phenylalanine or to liquefy Loeffler's inspissated horse serum. The strains were usually catalase positive, although weak and negative reactions were observed, mainly with Strains 1032A and V350. On the other hand, the tetra-methyl-p-phenyldiamine test for oxidase production was usually negative, although delayed and positive reactions were sometimes encountered, especially with these 2 strains.

Nitrate reduction was found to be a variable characteristic and was usually negative for nitrites when determined after 4 days incubation.

The production of hydrogen sulphide on blood tryptose agar slants was tested with filter paper strips impregnated with lead acetate. Weakly positive and negative reactions were encountered with equal frequency.

Inhibition of growth

No growth occurred on antibiotic serum dextrose agar (ASDA) or ASDA plus ethyl violet from any of the *A. seminis* strains. On the medium containing the lower concentration of thionin, reasonable growth was obtained from all strains except Strain V350. On the medium containing 1 : 100 000 basic fuchsin, Strains T981V, 70.64 and 6201 yielded a reasonable growth. With a few exceptions no growth was observed at any of the other concentrations or dyes used (Table 5).

TABLE 4 Results of biochemical tests conducted on strains of *A. seminis*

Biochemical test	Result
Carbohydrate breakdown:	
Oxidation/fermentation[1]	Negative
Monosaccharides[2]:	
Arabinose	Negative
Rhamnose	Negative
Xylose	Negative
Glucose	Negative
Fructose	Negative
Galactose	Negative
Disaccharides[2]:	
Sucrose	Negative
Maltose	Negative
Lactose	Negative
Trehalose	Negative
Polysaccharides[2]:	
Raffinose	Negative
Starch	Negative
Dextrin	Negative
Glucosides[2]:	
Salicin	Negative
Alcohols and Benzene derivatives[2]:	
Glycerol	Negative
Mannitol	Negative
Dulcitol	Negative
Sorbitol	Negative
Inositol	Negative
Other tests[2]:	
Methyl red	Negative
Voges-Proskauer	Negative
Litmus milk[3]	Negative
Selective or specific media:	
MacConkey agar[3]	No growth
Phenyl-alanine agar[2]	Negative
Malonate broth[4]	Negative
Simmon's citrate agar[4]	Negative
Gluconate broth[2]	Negative
Protein breakdown:	
H_2S production (Lead acetate strips)[5]	Negative or weakly positive
Indole production[4]	Negative
Gelatin liquefaction[3]	Negative
Urea hydrolysis (Eleks test)[4]	Negative
Proteolytic activity[2]	Negative
Other Enzyme activity:	
Nitrate reduction[2]	Negative, occasionally positive
Catalase[4]	Positive, occasionally negative
Oxidase[4]	Negative, occasionally positive

[1] According to the modified Hugh & Leifson method (Gibbs & Skinner, 1966)
[2] Cowan & Steel (1970)
[3] Difco Laboratories, Detroit, Michigan, U.S.A.
[4] Cruickshank (1965)
[5] Alton & Jones (1967)

All strains were found to be sensitive to penicillin, tetracycline, novobiocin and chloromycetin, partially resistant to erythromycin, neomycin and kanamycin and totally resistant to streptomycin (Table 6).

Comparison between A. seminis *and* B. ovis

In semen smears, *A. seminis* strains appeared as described above, whereas *B. ovis* was always more coccoid and arranged singly, rarely in pairs, but never in chains.

In smears prepared from cultures, organisms were non-acid-fast when stained with the Ziehl-Neelsen technique (Cruickshank, 1965). When stained with Stamp's modification of the Ziehl-Neelsen technique, *A. seminis* was decolorized with the weak acid. In semen smears where the modified method was employed, reactions similar to those in the culture smears were obtained, the only difference being that with *A. seminis* some organisms inside neutrophils occasionally retained the carbol fuchsin to a limited degree and consequently stained a faintly red colour. In both culture and semen smears stained with Hansen's technique, as described by Van Drimmelen (1960), *B. ovis* stained blue, while all the strains of *A. seminis* stained either red or reddish-purple (Table 1).

B. ovis did not grow under aerobic conditions, while growth, albeit poor, was obtained from all *A. seminis* isolates. With total replacement of air by carbon dioxide, poor growth was obtained with *B. ovis*, while 3 *A. seminis* strains showed poor growth and the remaining strains no growth at all. Both species grew better when incubated in a micro-aerophilic or carboxophilic atmosphere (Table 2).

From the results recorded in Table 3, it can be seen that growth of both *A. seminis* and *B. ovis* was either non-existent, poor, or irregular on the ordinary nutrient media, but better with those media normally used for more fastidious organisms. It will also be seen that the *A. seminis* Strains 1032A and V350 are more fastidious in their growth requirements than the other strains.

Except in the case of potato dextrose agar (pH 5,6), and possibly in that also of potato infusion agar, growth was always enhanced by the addition of blood or serum, although blood was always the better source of enrichment for both species.

TABLE 5 Inhibition of growth of strains of *A. seminis* and *B. ovis* on selective media containing various dyes

Selective medium[1]	Degree of bacterial growth						
	A. seminis strains						*B. ovis*
	T981V	70.64	6201	U207	1032A	B350	
ASDA[2]	—	—	—	—	—	—	++
ASDA & Ethyl violet	—	—	—	—	—	—	+
SDA & Thionin[3]:							
1: 100 000	+++	++	+++	+++	+	—	+++
1: 50 000	—	—	++	—	—	—	+++
1: 25 000	—	—	—	—	—	—	++
SDA & Basic fuchsin:							
1: 100 000	+++	+++	+++	—	—	—	+
1: 50 000	—	—	—	—	—	—	—
1: 25 000	—	—	—	—	—	—	—
SDA & Methyl violet:							
1: 100 000	—	++	+	—	—	—	—
1: 50 000	—	—	—	—	—	—	—
1: 25 000	—	—	—	—	—	—	—
SDA & Methylene blue:							
1: 200 000	—	—	—	—	—	—	—
1: 100 000	—	—	—	—	—	—	—
1: 50 000	—	—	—	—	—	—	—
SDA & Thionin blue:							
1: 100 000	—	++	—	—	—	—	—
1: 50 000	—	+	—	—	—	—	—
1: 25 000	—	+	—	—	—	—	—

[1] Preparation and test procedure according to Alton & Jones (1967)
[2] ASDA=Antibiotic serum dextrose agar
[3] SDA=Serum dextrose agar

TABLE 6 Antibiotic sensitivity of strains of *A. seminis* and *B. ovis*

Bacto unidiscs	Inhibition of bacterial growth[1]						
	A. seminis strains						*B. ovis*
	T981V	70.64	6201	U207	1032A	V350	
Penicillin (10 mcg)	++++	++++	++++	++++	++++	++++	++++
Streptomycin (10 mcg)	—	—	—	+	+	+	++++
Erythromycin (15 mcg)	++	+	++	++	++	++++	++++
Tetracyclines (30 mcg)	++++	++++	++++	+++	+++	++++	++++
Novobiocin (30 mcg)	++++	+++	++	++++	+++	++++	++++
Neomycin (30 mcg)	+	++	+	+	+	+	++++
Kanamycin (30 mcg)	+	+	+	+	+	+	++++
Chloromycetin (30 mcg)	++++	++++	++++	++++	++++	++++	++++

[1] Subjectively rated — to ++++ after 48 h incubation
++++=wide clear zone of no growth and — =uninhibited confluent growth surrounding discs

While *B. ovis* grew poorly on ASDA or ASDA plus ethyl violet, none of the *A. seminis* strains grew on either of these media (Table 5). Apart from Strain 6201, where a reasonable growth occurred at a concentration of 1: 50 000 thionin, all *A. seminis* strains were negative at both the higher concentrations while *B. ovis* grew reasonably well in the presence of all concentrations of this dye. Growth of all the organisms, with the exception of *A. seminis* Strains 6201 and 70.64, was inhibited by the other dyes used.

Whereas *B. ovis* was completely sensitive to the whole range of antibiotics used, *A. seminis* strains were either resistant or slightly sensitive to streptomycin, partly resistant to kanamycin and neomycin and, with the exception of Strain V350, also to erythromycin.

Discussion

The results of the bacteriological examination proved that on their morphological, staining, cultural and biochemical characteristics, all strains of *A. seminis*, with the exception of Strains 1032A and V350, conform to the descriptions given by Baynes & Simmons (1960), Livingston & Hardy (1964) and Worthington & Bosman (1968).

Whereas Worthington & Bosman (1968), in their comparative bacteriological studies, found that both the South African and the Australian organisms (Baynes & Simmons, 1960) reduced nitrates to nitrites, these studies in which the presence of residual nitrates was also determined proved that this was a variable characteristic, not only between cultures but also on subsequent tests and on re-examination of

the same strain. It was found that with the methods of detection used in this study, hydrogen sulphide production was also a very variable feature, which nevertheless produced only a weak reaction. Some differences were also found in catalase and oxidase production, but since these variations occurred rather infrequently and predominantly in strains similar to 1032A and V350, these organisms can generally be regarded as catalase positive and oxidase negative.

From previous descriptions and taking into consideration the findings of this study, it seems likely that the Gram-negative pleomorph described by Dodd & Hartley (1955) could have been *A. seminis*, although *H. ovis* described by Roberts (1956) and subsequently by Kater, Marshall & Hartley (1962) and Claxton & Everett (1966), appears to be different. Strains 1032A and V350 appeared to follow more closely the description of *H. ovis* as given by Roberts (1956). The possibility also remains that the unidentified Gram-negative organism isolated from a few cases of traumatic epididymitis in young Dorset Horn and Poll Dorset rams by Pulsford, Eastick, Clapp & Roberts (1967) could have been *A. seminis*. Ekdahl, Money & Martin (1968) reported that a substantial percentage of Gram-negative pleomorphs and *Actinobacillus*-like organisms was isolated from epididymal lesions of rams and that these organisms were not identical with the *A. seminis* strain described by Baynes & Simmons (1960). Since a description of their bacteriological properties was not given, it is possible that they could have been different strains of *A. seminis*.

The inclusion of *B. ovis* in the tests conducted on strains of *A. seminis* was considered desirable to differentiate between these 2 organisms commonly found in ram semen. For practical purposes these tests were therefore limited to morphological characteristics, staining reactions, atmospheric requirements and growth patterns and included antibiotic and dye sensitivity.

On both culture and semen smears, *B. ovis* can be distinguished on its more coccoid form and single arrangement. With the modified Ziehl-Neelsen technique (Stamp, McEwen, Watt & Nisbet, 1950), *B. ovis* stains weakly acid-fast, while *A. seminis* is decolorized by the weak acid and stains blue. In semen smears stained by this method, *B. ovis* is always clearly discernible, while in acute clinical cases *A. seminis* organisms could not always be demonstrated, although cultural examination of the same semen sample would yield a pure and abundant growth.

Growth of *B. ovis* is always tardier, even on the most favourable media. After 48 h a greyish-white growth of *B. ovis* was just visible to the naked eye, while individual colonies were still indistinct compared with the well-developed "dewdrop"-like or differentiated colonies of *A. seminis*. While all strains of *A. seminis* could be isolated under aerobic conditions on primary culture, this was not possible in the case of *B. ovis*. Since growth of *B. ovis* but not *A. seminis* took place on ASDA, ASDA plus ethyl violet or SDA plus thionin at the higher concentrations, these media were found to be suitable for differentiating these organisms.

Although *Pasteurella haemolytica* (untypable strains) has occasionally been isolated from ram semen, its aetiological importance has not yet been determined (Cameron, 1972). However, this organism can be differentiated from *A. seminis* on account of its morphology, colony formation, haemolytic properties and sugar reactions.

Various other Gram-negative pleomorphs, which as yet defy identification, are also occasionally found in ram semen but, since they differ markedly from *A. seminis* in their bacteriological characteristics, they should not easily be confused. Recently *Actinobacillus actinomycetemcomitans* was isolated from the testes of 23,4% of rams with palpable lesions in Idaho and East Oregon (Delong, Waldhalm & Hall, in press) while M. Laborde (Centro De Investigaciones Veterinarias "Miguel C. Rubino", Pando, Uruguay, personal communication, 1978) also isolated a hitherto unidentified Gram-negative pleomorphic organism from the semen of a ram with epididymitis.

Since on their bacteriological characteristics the organisms classified as *A. seminis* at present clearly fall within 2 groups and these strains also differ markedly from accepted members of the genus *Actinobacillus*, a full comparative taxonomic study of the ovine actinobacilli seems to require attention. Evidence to this effect is also presented by both Gumbrell & Smith (1974), who studied the deoxyribonucleic acid base ratios of a number of ovine actinobacilli and compared them with other members of the genus *Actinobacillus*, and by Van Tonder (to be published), who investigated a possible antigenic relationship by using the complement fixation technique. Bergey's manual of Determinative Bacteriology (Buchanan & Gibbons, 1975) also states that, on account of its failure to ferment sugars, *A. seminis* should be excluded from the genus *Actinobacillus*.

Conclusions

1. Exhaustive growth studies and biochemical tests have proved that *A. seminis* has defined morphological, staining, cultural and biochemical properties.
2. Strains T981V, 70.64, 6201 and U207 conform to the earlier description of Baynes & Simmons (1960), while strains 1032A and V350 more closely resemble *H. ovis* described by Roberts (1956).
3. *B. ovis* and *A. seminis* can clearly be distinguished on their morphology, Stamp-staining reaction of both semen and culture smears, colony morphology, the delayed development of growth of *B. ovis* and sensitivity to dyes and antibiotics.

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